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Production of a variant of encephalomyelitis virus by its adaptation to extraneural tissue.

by K. O. Habermehl and W. Diefenthal.

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The composition of a viral population is governed extensively by the selective influence of the environment. Differences in the host species, the cell type and the mode of infection lead to selection of viruses with special or novel properties, expressed in such factors as the complex concept of pathogenicity. The virus of mouse encephalomyelitis (TEM) described by Theiler (1) is marked by a characteristic pathogenicity even under natural conditions. Aside from the original TO strain, strain GD VII is also noted for particular virulence.

TEM deviates from other neurotropic viruses by its transmissibility almost exclusively by direct inoculation of the central nervous system (Theiler and Gard 2,3). The occurrence of latent infections of the intestinal tract without manifestations (Olitsky 4) shall be ignored in this connection. While the absence of manifest symptoms after extraneural application of the virus may conceivably prevent the appearance of neurotropy, comparative studies of TEM on different types of tissue in vitro reveal a distinct propagation only in the brain tissue.

The utilization of species-specific, mesenchymal cells was chosen with the aim of examining the behavior of a strictly neurotropic virus after adaptation to extraneural tissue.

Methodology.

Tissue cultures.

Mouse fibroblasts: The tissue cultures of embryonal mouse fibroblasts were prepared from eventrated mouse embryos by the customary trypsination technique (Dulbecco 5, Youngner 6, Rappaport 7). Cells of the first subculture obtained by trypsination were utilized. For additional methodical details, see Diefenthal and Habermehl (8).

Liver and kidney epithelium, heart fibroblasts: These tissues were grown in roll cultures on plasma film. The use of about 40 tissue particles from embryonal organs produced a large yield of cells. The mature cells were loosened with trypsin and continued their growth as subcultures in Petri dishes on plasma film.

Brain tissue: The same process was used as described in the preceding paragraph, although cell passages could not be carried out.

MS 37: The ascitic form of mouse sarcoma 37 was utilized; the line had been maintained through 200 passages as a solid transplantation tumor. The tumor cells obtained from the ascites were grown directly on the glass.

L strain cells (Earle): These cells came from the clonal strain NCTC B 929-323-303 which had been grown directly on glass in continuous passages.

Nutrient medium: All cells were grown on the following medium: 10 to 20% calf serum and 80% Earle's solution with lactalbumin hydrolysate and yeast extract in terminal concentration of 0.5% or 0.1% with addition of 100 units of penicillin and 100 γ of streptomycin per milliliter. The medium was changed every 2-4 days.

Composition of the agar mixture: 12 parts 2.7% "Difco agar purified," 6 parts 6 times concentrated lactalbumin hydrolysate and yeast extract solution in twice-distilled water, 8 parts 4 times concentrated Earle's solution without sodium bicarbonate, 6 parts sodium bicarbonate (0.88 g in 75 ml twice-distilled water), 15 parts nutrient medium without addition of serum (see above), with admixture of antibiotics. Sterilization of agar in the steaming kettle, that of the remaining ingredients by Seitz filtration.

Virus.

The utilized virus strain is described in detail in the first chapter of the "results."

Titer determination.

Plaque counts in monolayer cultures: The examined virus was diluted by decimal powers with nutrient medium without addition of serum. Inoculation of two Petri dishes with 0.3 ml of the appropriate viral dilution. Adsorption time 50 min at 37°C. After removal of the inoculum by suction, superimposition of 3 ml agar mixture at 42°C. The dishes were stained with diluted (1:3,000) neutral red solution in phosphate-buffered saline (PBS) four days after inoculation and the plaques were counted.

Determination of MLD_{50}: Viral dilution as above. Intracerebral inoculation of 8 mice (weight 9-11 g) per dilution. Observation for 4 weeks. Computation of MLD_{50} according to the method of Reed and Muench (9).

Results.

I. Characterization of the original strain Theiler GD VII used for adaptation.

The starting material consisted of a brain, infected intracerebrally with the GD VII strain of Theiler's mouse encephalomyelitis virus (TEM), preserved in 50% glycerin-Na Cl solution, received from R. Rustigian, Univ. of Chicago, in February 1953. The latter institute had carried out 29 intracerebral passages. The number of previous passages was unknown. We conducted 8 cerebral mouse passages, partly in marginal dilutions. Changes relative to the pathogenicity, clinical course and virus titer did not occur during these passages.

a) Mode of inoculation. In order to characterize the pathogenicity of TEM, the results of different modes of inoculation were compared. As evident from Table 1, infections were consistently produced by intracerebral or intraspinal injection, whereas no signs of infection were noted after the utilization of other modes. There was one exception, in which intramuscular injection was followed by limp paresis of both hind legs.

b) Clinical course. Depending on the infective dose instilled intracerebrally, the first symptoms of encephalitis appeared at the earliest on the 3rd day (see below). The animals became restless, agitated. Later, equilibrial disturbances, tonic-clonic cramps were seen, during which the animal frequently died. In addition, trotting movements around the vertical axis in the direction of the focus of infection were noted. Death usually occurred in a characteristic position with over-extended posterior extremities. Not one of the 2,000 animals infected intracerebrally with the non-adapted original virus recovered; all apparent infections terminated in death. There were only a few animals that showed limp paresis of the posterior extremities in addition to encephalitis with this mode of injection. The rate of paralysis upon intracerebral injection amounted to 4% (numerical ratio of paralysis: encephalitis). Intraspinal injection was followed by limp paresis of both hind legs with an ascending tendency, accompanied by encephalitis.

c) Latent period and virus titer. The duration of the latent period until the appearance of symptoms proved to be dependent upon the dosis (10). The reciprocal value of the median latent time expressed in days was directly proportional to the negative logarithm of the viral concentration in the inoculum (Fig. 6). This finding may be reproduced repeatedly. However, the determination of MLD_{50} and its evaluation according to Reed and Muench (9) has proved to be better suited to the titration of virus in the cerebral substance than the determination of the latent period.

The starting material for adaptation consisted of the brains of 14 mice showing signs of severe encephalitis between the 3rd and 5th day after intracerebral inoculation. The animals were killed and the 10% brain suspension in Tyrode solution was frozen for storage purposes at -70°C. The viral content of this suspension amounted to $7.9 \cdot 10^7$ MLD_{50} per gram of brain.

d) Behavior of the unadapted virus strain in tissue culture. Reproduction of TEM in tissue culture was possible only in roll cultures of embryonal mouse brain tissue. We used roll cultures in which about 40 pieces of cerebral tissue had been started on plasma film; their growth zones touched owing to their expansion, placing a nearly gapless mat of cells on the glass wall. The dosis of inoculation was 100 MLD_{50}. Repeated tests under these conditions showed a low rate of propagation (Fig. 1). The yield of virus reached its apex on the 4th day p.i., followed by continuous reduction. A distinct cytopathogenic effect (CPE) could not be observed. It must be remembered in this connection that the roll cultures of embryonal brain tissue contained, in addition to the parental fragments, primarily sprouting neurites and cells resembling fibroblasts, which must be classified as glia-supporting tissue. A CPE possibly present in the nerve cells of the parental particles could not be evaluated, although it is conceivable that reproduction had occurred there. The neurites do not necessarily show a CPE with sufficient clarity; moreover, our present experience makes it likely that no reproduction takes place in the cells of the cerebral supporting tissue.

No CPE was observed in the following types of tissue under variable conditions of culture: 1. Embryonal mouse liver, a) in roll cultures, b) as a monolayer culture of the first cell passage on plasma film in Petri dishes, 2. embryonal mouse kidney, a) in roll cultures, b) as monolayer culture of the first cell passage on plasma film in Petri dishes, 3. mouse heart fibroblasts in roll cultures, 4. embryonal mouse fibroblasts of the first cell passage in Petri dishes or Porter flasks, growing directly on the glass, 5. ascitic cells of mouse sarcoma 37 (MS 37), growing directly on glass in upright tubes, 6. L strain cells (Earle), NCTC 929, growing on the glass of Porter flasks.

II. Adaptation of strain GD VII to mouse fibroblasts.

Adaptation was made to embryonal mouse fibroblasts of the first cell passage grown on glass in Porter flasks. An inoculum of $4 \cdot 10^5$ MLD_{50} was used in the initial virus passage. After an adsorption period of 4 hours at 37°C, the inoculum was drawn off and fresh nutrient was added. No CPE was observed four days later. The cells were scraped from the glass wall and, together with the tissue culture medium, were treated with ultrasonics for 3 minutes. Inoculation of the second passage was carried out with 4 ml of this suspension. Up to the 7th passage, inoculation, adsorption and incubation proceeded in the same manner; only the destruction of cells was changed to vigorous shaking with glass beads

from the second passage on in order to avoid thermic inactivation during exposure to ultrasonics. Starting with the 6th virus passage, destruction of cells was followed by centrifugal removal of coarse cell constituents (10 min at 2,500 RPM). No cytopathogenic lesions were seen up to this instant. Each passage was accompanied by a test of pathogenicity for animals through intracerebral inoculation of 4 mice with undiluted culture suspension. The results obtained here will be discussed later.

Since the culture of the 8th virus passage was contaminated with fungi, we retracted to a brain infected with the 7th culture passage (7/1), which was subjected to renewed cerebral passage with addition of moronal (7/2); a 5% cerebral suspension of this passage was then used in further inoculation of tissue cultures. Subsequent virus passages through tissue culture occurred as described above. First signs of commencing CPE were noted during the 9th virus passage (9/2). On the 3rd day after inoculation there were isolated, circumscribed areas in which the cells had assumed a round shape; this degenerative manifestation increased on the 4th day; a portion of the cells separated from the glass wall. Nearly identical changes were noted during the 10th virus passage (10/2). The tissue culture used in the 11th virus passage (11/2) was marked by particularly dense cell growth; on the 3rd day after inoculation the entire cellular film separated from the glass wall in large membranes (in contrast to the non-inoculated control culture), preventing the evaluation of manifestations of cellular degeneration. The 12th virus passage (12/2) revealed a distinct CPE of all cells two days after inoculation. The cells were rounded off and showed partial granular decomposition. The nuclei were pycnotic; a large portion of cells revealing this form of change separated from the glass wall (Fig. 3 and 4). Starting with this virus passage, all subsequent passages disclosed an identical, typical CPE. Later, only the tissue culture medium was used for passage of the virus.

Upon inoculation of monolayer cultures in Petri dishes with decreasing dilutions of tissue culture fluid, cytopathogenic changes graduated in typical fashion were demonstrated after superimposition of agar, ranging from a confluent CPE to isolated plaques (Fig. 5).

It was noted occasionally during adaptation that the CPE initially became weaker in the course of two passages, and then disappeared altogether. Hallauer (11) observed similar behavior during adaptation of fowl cholera virus. In such cases a return was made to a passage, usually the penultimate, which still had shown a distinct CPE. The renewed passage presented no difficulties. The cause of this effect may possibly be found in changeable cultural conditions. Even a slightly excessive age of the utilized tissue culture or an alteration in the composition of the medium had a marked influence on the development of the CPE and on viral yield.

The described difficulties encountered in virus passage may be ascribed to the fact (other than the assumption of inconstant test conditions) that the virus had undergone a genotypical or phenotypical change. For this reason and in order to exclude the possibility that the resultant adaptation only represented a phenotypical adaptation, 12 additional virus passages were carried out in tissue culture after the appearance of the first CPE. A CPE appeared regularly on the 2d to 3rd day after inoculation in subsequent passages.

Plaque-purification of the adapted viral strain. As already indicated above, inoculation of monolayer cultures with an appropriate viral dilution and subsequent superstratification with an agar mixture produced clearly identifiable plaques (Fig. 5). The plaques were sharply delineated from their environment and showed an average diameter of 3 mm 4 days after inoculation. It was noted occasionally that the size of plaques within one Petri dish was subject to fluctuation. Attempts to isolate variants from them have had negative results until now, since it could not be proved in this case that the size of plaques represents a hereditary property.

In the isolation of a plaque-purified virus strain, a cylinder was cut from the area of a plaque with the aid of a glass pipette with an internal diameter of 1 mm, resuspended and inoculation on new monolayers in decreasing dilutions. Only those plaques were chosen that were at least 2 cm apart from neighboring plaques. Whenever possible, we used dishes that contained only 1 or 2 plaques. Three successive plaque passages were carried out in the manner indicated. This was followed by a passage through Porter flasks with fluid nutrient for enrichment of the virus.

Based on the number of passages in vitro and in vivo, the adapted virus strain received the designation Theiler GD VII (26/2). Titration of strain 26/2 in monolayer tissue cultures showed $6.6 \cdot 10^6$ pfu/ml (plaque forming units).

III. Characterisation of the adapted virus strain.

The adapted strain differed from the original by a number of properties, which are listed below.

a) Cytopathogenicity in the tissue culture. Strain 26/2 of TEM leads to the aforementioned CPE in tissue cultures of embryonal mouse fibroblasts and L strain cells, beginning on the 1st day, with regular appearance on the 2d day after inoculation and completion on the 3rd day. The cytopathogenic lesions appear a little later on L strain cells and are not as pronounced as in embryonal mouse fibroblasts. Cultures of other types of tissue were not examined.

b) Behavior of strain 26/2 in animal tests. The adapted virus revealed considerably attenuated pathogenicity for mice. Titer determination by means of intracerebral inoculation gave a value of $7.9 \cdot 10^1$ MLD_{50} per ml tissue culture fluid. At a level of $6.6 \cdot 10^6$ pfu/ml in the culture fluid, the attenuation of pathogenicity amounts to a magnitude of 1:100,000.

It was noted during experimental titer determination of strain 26/2 that mortality was not limited to high viral concentrations. Greater dilutions below LD_{50} caused the death of relatively more animals than in the case of unadapted virus.

The change in pathogenicity was also noticeable by a distinct prolongation of the latent period. Fig. 6 shows the latent time in days as a function of the viral dilution. The curve indicates that undiluted culture fluid of strain 26/2 produces a latent period that is achieved by unadapted virus only in dilutions of about 10^{-7}. This fact again points to differences in pathogenicity of a magnitude comparable to the titer differences in vivo indicated above.

Aside from pathogenicity, the clinical picture has also changed. As mentioned in Chapter I relative to the characterization of original virus, the rate of paralysis is 4%. The adapted virus, on the other hand, reveals a rate of paralysis of 50% in repeated controls. It is particularly noteworthy that strain 26/2 usually induced a pure paralysis without encephalitic symptoms, whereas the few cases of paralysis in connection with unadapted virus were dominated by encephalitis. Finally, the fact bears mentioning that a few cases of paralytic affliction induced by infection with strain 26/2 gave evidence of healing of defects, with permanent paresis of one extremity.

In the course of further characterization of the adapted strain's pathogenicity, the results were compared with those of the unadapted parental strain (Tab. 1). A marked difference was seen only in the case of intracerebral and intraspinal modes of infection. The other paths of inoculation produced no divergences.

c) Change in pathogenicity in the process of adaptation. The virus content of the various passages was determined in the course of adaptation. At the same time, the extent of changes in pathogenicity was established by examination in vivo and in vitro. The results are reflected in the schematic representation (Fig. 2).

Titer determinations in vitro. The determination of titer became possible in vitro upon the appearance of the initial CPE. Table 2 depicts the results.

Titer determinations in vivo. Virus passages in tissue cultures were accompanied partly by spot checks for pathogenicity, partly by titer determinations through intracerebral inoculation. The results are listed in Table 3.

d) Demonstration of the presence of a genotypical adaptation by the interpolation of animal passages. In order to confirm the assumption that the accomplished adaptation is an expression of change in the genotype of the virus particle, we afforded the virus an opportunity to propagate under original environmental conditions.

1. Interpolation of an animal passage. Proceeding from passage 14/2, marked by a content of $1 \cdot 10^3$ pfu/ml and an MLD_{50} of $10^{-4.91}$/ml, an animal passage was carried out by intracerebral inoculation of diluted (10^{-1}) tissue culture fluid. The virus suspension obtained from the brain of a mouse infected by this inoculum (14/3 G) was subjected to 2 successive passages through tissue culture (15/3 and 16/3). The titration of viral content in vitro is shown in Table 4.

2. Interpolation of several animal passages. Starting point was passage 19/2, marked by a viral content of $3.5 \cdot 10^5$ pfu/ml; pathogenicity was determined from the mortality of 2 out of 4 inoculated animals (concentration of inoculum: 10^{-1}). First, one animal passage (19/3 RM) was accomplished through intracerebral inoculation of diluted (10^{-1}) tissue fluid. The spinal cord of a mouse falling ill with limp paresis of both hind legs was removed and the virus (19/3 RM) in the form of a 2% tissue suspension was subjected to 2 successive tissue culture passages (20/3 and 21/3). These were followed by 7 successive animal passages (21/4 RM to 21/10 RM). During this process, the spinal cord of a mouse showing signs of limp paresis of the hind legs was injected intracerebrally in every case in the form of a 2% suspension. The results of titer determinations of this test series are found in Table 5.

e) Neutralization test. The identification of the adapted virus strain was accomplished by the neutralization test. Rabbits were immunized with 6 injections of strain 26/2 over 2 months. Controls were established with normal rabbit serum and with phosphate-buffered saline (PBS). The sera were inactivated for 30 min at 56°C just prior to use. It proved practicable to test diminishing viral concentrations at a constant serum dilution of 1:8. Upon the action of strain 26/2 antiserum on unadapted parental virus for 1 hour at 37°C, intracerebral inoculation of mice produced the results listed in Table 6.

Investigation of the neutralizing effect of anti-26/2 serum on the virus of strain 26/2 in tissue cultures yielded results of the same magnitude.

Discussion.

The course of illness following intracerebral infection of mice with Theiler's encephalomyelitis virus, strain GD VII (TEM) corresponds both clinically and pathologic-anatomically to the picture of an infection with poliomyelitis virus. An extensive agreement of both clinical pictures is noted also in rarely occurring spontaneous cases. The particles of both viruses reveal similarities with respect to size and structure (Melnick 12). Theiler and Gard (2) have therefore proposed TEM as a model for studies in the field of poliomyelitis. Limitations are imposed, however, by differences in antibody formation (Pette 22) and infectivity to the extent that TEM is transmissible almost exclusively by intracerebral or intraspinal inoculation. There are isolated reports of encephalomyelitides following intranasal, intraperitoneal or intramuscular inoculation with extremely high dosages (Theiler and Gard 2, Gildemeister and Ahlfeld 13). Peroral inoculations usually did not produce symptoms of central nervous system affection (Olitsky 4, H. v. Magnus 14,15). The TEM strain used here (GD VII) showed a high pathogenicity upon intraspinal and intracerebral inoculation; in spite of instillation of high viral dosages, intramuscular inoculation produced only one case of paralysis. Other routes of inoculation failed to induce pathogenicity. Rustigian (16) produced symptoms at the rate of 100% by intramuscular inoculation, and Theiler and Gard (2) observed paralyses after intraperitoneal infection. The rate of paralysis represented another deviation from references found in the literature. Whereas Theiler (1) described a preponderance of paralyses and Rustigian (16) listed 29%, we noted them in only 4% of the cases. The duration of latency was dependent on the dosage, in agreement with Gard (10). The viral content of the brain substance corresponded to values found by Rustigian (16), Gard (10) and Theiler (1,2). Dependence upon the route of inoculation suggests that TEM is a strictly neurotropic virus. This assumption is supported by the fact that TEM cannot be grown in vitro on homologous, extraneural tissue, where divergence from poliomyelitis virus is particularly characterized by the circumstance that no reproduction and no CPE can be achieved on monolayer cultures of mouse kidneys without prolonged adaptation (Falke 17). As we were able to demonstrate, neither a CPE nor propagation was attainable on liver, kidney, embryonal fibroblasts, L strain cells or tumor cells (MS 37). In vitro propagation of TEM in mouse ependymoma cells, as observed by Pearson (18), is contrasted with aforesaid findings to the extent that the resultant viral yield was small and no CPE appeared (personal communication). Studies by Franklin, Wecker and Henry (19,20) of a mouse encephalomyelitis virus not further characterized, showed a primary CPE on L strain cells and infectivity upon subcutaneous inoculation; the question whether this virus is identical with Theiler's strain remains unresolved. We were able to demonstrate reproduction only in roll cultures of embryonal mouse brain, with a relatively low yield of virus. The GD VII strain used by us revealed divergences from the pathogenicity described by Theiler and Gard (1,2), as already noted. It is possible that an encephalitogenic variant has developed due to numerous brain passages carried out in the meantime (Kearney 21).

The indicated findings relative to pathogenicity and tropism point to a special position of TBM virus. From this viewpoint it would be of particular interest to investigate the behavior of this virus after adaptation to homologous, extraneural tissue. Based on the experiences of Burnet (25), Sabin (23,24) and Hallauer (11), the adaptation of strain GD VII to embryonal mouse fibroblasts was accomplished from the start in the form of "rapid passages." The execution of passages in rapid succession, with a high inoculum, caused the first appearance of a CPE after the 9th passage in vitro, a regular CPE after the 12th passage. Following the 22nd passage, the virus was subjected to three successive plaque purifications and enriched by an additional passage. The adapted virus strain, designated "26/2", was characterized by high pathogenicity for mouse fibroblasts and gave a corresponding tissue culture titer of $6.6 \cdot 10^6$ pfu/ml. The morphological changes effected by strain 26/2 in tissue cultures was completely identical with the CPE evoked by poliomyelitis virus. Adaptation was accompanied by attenuation of pathogenicity for animals upon intracerebral and intraspinal inoculation, amounting to 1:100,000; pathogenicity remained unchanged in connection with other routes of inoculation. The altered pathogenicity was further characterized by an increase in the rate of paralysis from 4 to 50%, a peculiarity already observed by Rustigian (16) during the adaptation of Theiler virus to eggs. An additional factor speaking for the attenuation of pathogenicity was given by prolongation of latency.

Occasional occurrence of defect restitution following intracerebral inoculation with strain 26/2 represents another proof of attenuation. Sabin (23) observed a few cases with mild clinical symptoms or only histologically demonstrable affection of the spinal cord, without paralysis, after inoculation of monkeys with attenuated poliomyelitis strains. These findings led to the conclusion that the attenuated virus, having induced a local infection of the spinal cord, was unable to spread, and the disease process was limited to such an extent that no clinical symptoms could appear.

A closer examination of the individual adaptive stages determined that the content of plaque-forming units remained quite constant from the 11th to the 26th tissue culture passage, with one exception of unexplained origin.

The characteristic pathogenicity of TBM, expressed, among others, by the strict neurotropy, gave rise to questions about the behavior of these properties during the course of adaptation to homologous, extraneural tissue. The concept that the adaptation of virus to heterologous tissue is due to the selection of a variant with poor chances of survival in the previous host, readily explains why the resultant variants reveal differences in the host spectrum and, consequently, attenuations in pathogenicity. As was demonstrated, adaptation to a homologous, mesenchymal tissue also led to reduction in pathogenicity, without the existence of species-specific genetic structural variations

in the host. Despite the newly acquired properties of pathogenicity for mesenchymal tissue, the use of different routes of inoculation, including the arterial, failed to effect an infection of the central nervous system. It seems that the penetration of the hemal-cerebral barrier is of decisive significance (Bodian 26). As a supplemental factor, it should be noted that the residual neuro-pathogenicity of strain 26/2 in the utilized dosages was sufficiently great to make an affection of the central nervous system visible. Sabin (23) achieved attenuation of the pathogenicity of poliomyelitis virus by continuous passages through kidney cultures of Cynomolgus monkeys. Considering the fact that the utilized polio strains were highly virulent for Cynomolgus monkeys, it follows that the attenuated variants had again developed on homologous tissue. Enders, Weller and Robbins (27) isolated an avirulent polio variant by cultivation on extraneural, human tissue.

When the adapted virus was subjected to several successive cerebral passages, it was shown that the properties newly acquired in the tissue culture were retained even under the original environmental conditions. Both this fact and the cytopathogenic effect suddenly arising after 9 blind passages support the assumption of a genotypical adaptation. The changed reactive norm of the genotype could be due to a previously extant mutant, a mutant accidentally developed during adaptation, or to segregation.